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A FILAMENTOUS BACTERIUM ON THE BRINE SHRIMP AND ITS CONTROL[1]

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ABSTRACT A strain of a colorless, filamentous bacterium (tentatively identified as *Leucothrix mucor*) heavily infests the brine shrimp, *Artemia salina*. Its ultrastructure, unlike that of some other strains, does not reveal a distinct middle layer between its outer cell wall layer and cytoplasmic membrane, irregular blebs extending from the cell layers, or an external sheath. An entire infestation, represented as a mat of the bacterium with associated debris and microorganisms, sloughs from the shrimp when exposed to a variety of treatments. Primarily because most effective treatments are toxic to the shrimp, 100 ppm terramycin provides the treatment of choice.

INTRODUCTION

A bacterium, tentatively identified as a strain of *Leucothrix mucor*, infested 100% of the adult brine shrimp, *Artemia salina*, in a 200-liter intensive culture tank. These heavily infested shrimp died at a faster rate than the stock could be replaced by maturing individuals. Death, however, did not appear to occur rapidly upon infestation. Because of the vulnerability of the shrimp to the bacterium, the known pathogenic effect of *L. mucor* on many crustacean larvae and eggs confined in rearing facilities (e.g., Nilson et al. 1975, Lightner 1975), and the potential to contaminate larval crustaceans by feeding them brine shrimp, we tested a variety of treatments on infested individuals. Also, because of the large number of poorly characterized strains of *L. mucor*, we present some morphological data on the form we encountered.

MATERIALS AND METHODS

Infested *Artemia salina* were obtained from the Oyster Biology Section of the Gulf Coast Research Laboratory. Brine shrimp eggs, presumably uninfested, came from San Francisco Bay ponds and the hatched shrimp were maintained in salinities of 45 to 50 parts per thousand (ppt) at 23 to 25°C with whole wheat flour; adults averaged 11 mm in total length.

In order to identify the bacterium, we observed it with a Nomarski differential interference contrast and an electron microscope, studied it histologically, and cultured it using the methods of Pringsheim (1957). A few heavily infested brine shrimp were embedded in paraffin and sectioned at 6 to 7 μm, and the sections were stained using Harris' hematoxylin and eosin stain, Bennhold's method for amyloid, McManus' method for glycogen (PAS), and alcian blue method for mucosubstances (Luna 1968). Additional material was fixed in 3% glutaraldehyde, post-fixed in osmium tetroxide, embedded in Spurr's embedding medium, sectioned on an LKB ultratome, stained with uranyl acetate and lead citrate, and photographed with a Siemens Elmiskop 1A electron microscope.

Infested shrimp in groups of about 15 individuals were placed in glass bowls each containing 70 ml of artificial seawater (30 ppt, Rila Marine Mix) at 24±1°C. Diseased shrimp were exposed to the various chemicals listed in Table 1. All tests were conducted in duplicate or triplicate, usually for periods of time commonly used for each type of treatment. When treatments lasted 48 hours, saltwater and chemicals were replaced after 24 hours. Experimental hosts were not fed while tested, and we defined cure as the absence of bacterial filaments.

RESULTS

Bacterium

The colorless, filamentous bacterium attached its PAS- and congo red-positive holdfast to the gills, swimming appendages, antennae, and all other external surfaces of the shrimp's adult and late instar stages. The site of attachment stained purplish-red to purple using McManus' method and predominately blue using the alcian blue method. A loose mesh of bacterial filaments helped trap and support considerable debris and a variety of unidentified microorganisms (Figures 1–4). Rosettes of filaments occurred commonly (Figure 5). Some infested appendages exhibited extensive deterioration, but the histological relationship between the bacterium and those lesions was not critically examined. Most attached filaments did not appear to penetrate deeply into the cuticle, and we never observed filaments extending through the cuticle or within a host.

Filaments varied in appearance. Most typically contained refractive granules (Figure 4); however, many filaments had few or no granulated segments. We did not analyze the chemical composition of these granules. Widths of 50 typical

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TABLE 1.

Treatments tested for controlling infestations of *Leucothrix mucor* on adult brine shrimp.

Treatment	Number of shrimp	Number of replicates	Concentration[1]	Exposure-period in hours	Average percentage cured ± SE	Average percentage died ± SE
Control	152	9	—	48	25.5 ± 2.5	34.2 ± 6.9
Formalin	46	3	40 ppm	12	58.7 ± 5.2	21.7 ± 7.3
Potassium permanganate	45	3	10 ppm	1	44.4 ± 4.7	20.0 ± 3.1
Nitrofurazone[2]	46	3	100 ppm	6	47.8 ± 3.8	10.8 ± 2.8
Cutrine[3]	45	3	100 ppm	4	60.8 ± 3.2	15.2 ± 1.4
Cutrine	45	3	100 ppm	48	28.8 ± 6.5	53.3 ± 3.9
Cutrine	45	3	0.5 ppm	4	51.1 ± 1.8	20.0 ± 3.1
Cutrine	47	3	0.5 ppm	48	48.2 ± 7.3	48.9 ± 8.8
Terramycin[4]	78	5	10 ppm	48	37.1 ± 5.5	38.4 ± 3.1
Terramycin	77	5	50 ppm	48	45.4 ± 7.2	36.3 ± 6.7
Terramycin	74	5	100 ppm	48	67.5 ± 8.1	25.6 ± 4.6
Terramycin	84	5	200 ppm	48	51.1 ± 2.2	21.4 ± 6.1
Terramycin	16	1	200 ppm	1	43.8	12.5
Salinity reduction[5]	47	3	10 ppt	48	72.3 ± 9.3	19.1 ± 8.8
Freshwater	15	1	0 ppt	1	40.0	33.3

[1] Based on commercial preparations and not active ingredients
[2] 5-nitro-2-furaldehyde semicarbazone
[3] Copper sulfate + tricthanolamine and other additives
[4] 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-nephthacenecarboxamide
[5] Produced with Rila Marine Mix

filaments from three shrimp ranged between 1 and 2 μm. Cells near the base of the filaments averaged 2.3 μm (1.0 to 2.9 μm) long by 1.8 μm (1.5 to 2.0 μm) wide, those near the middle were 1.6 by 1.6 μm, and those near the apex, 2.2 by 1.0 μm. Twenty nongranulated cells near the middle of a filament averaged 2.4 by 1.3 μm.

Examination of the filament's ultrastructure revealed fibrillar nuclear material, storage granules, and ribosomes dispersed in the cytoplasmic matrix. All those features (Figures 6–11) are considered typical components of *L. mucor* in its broad sense, except for an aspect presented in Figure 8 and mentioned below. The outer wall layer and the cytoplasmic membrane were both simple and smooth. Each was about 11 nm wide with the total wall about 45 to 55 nm wide.

Some filaments appear similar except the cells obtain a length as long as ten times the width. Figure 8 illustrates the variation in length between adjacent cells of these filaments. The long cell length is not typical of *L. mucor*, and the organism probably represents a strain or species distinct from the dominant organism of our study.

The cuticle at the site where *L. mucor* attached (Figure 7) was altered and notably rougher than the smooth adjacent cuticle. No significant underlying cellular damage was apparent, even though slight penetration of the holdfast occurred within the cuticle.

The second cell in the middle filament of the rosette in Figure 7 represents one of several similar examples observed; it differed from the others shown by having more granules and a large, central, irregularly shaped, compact structure. The cell is possibly a reproductive cell; however, we do not discount the possibility of a normal cell undergoing degeneration.

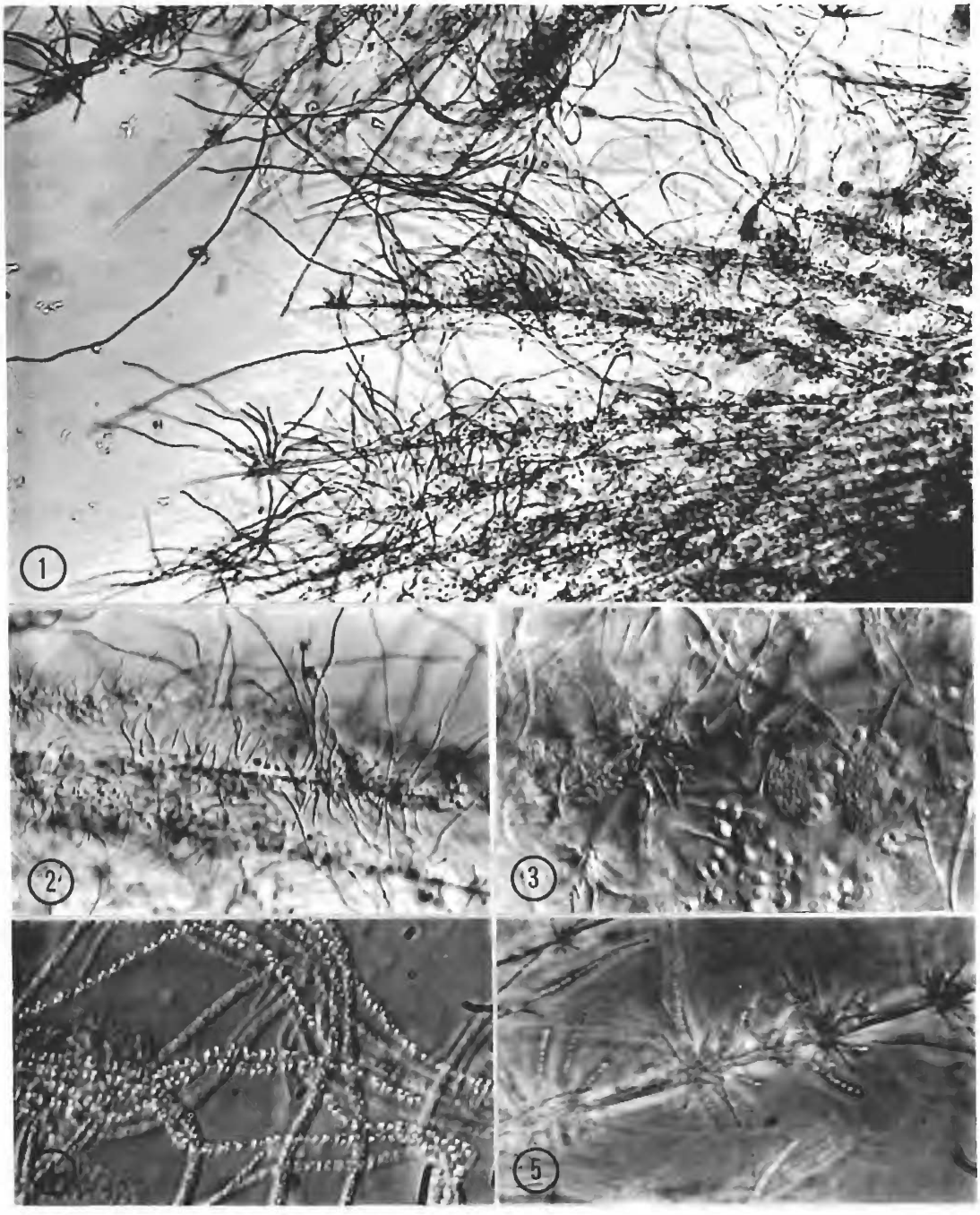
Nauplii had no conspicuous infestations, whereas juveniles at about the sixth instar, the stage when the rate of molting frequency decreases, possessed numerous short filaments, both isolated and in rosettes.

A moderately heavy infestation was conspicuous when comparing an infested host with a noninfested shrimp (Figures 9–10). The entire bacterial mass sloughed from treated individuals (Figure 11).

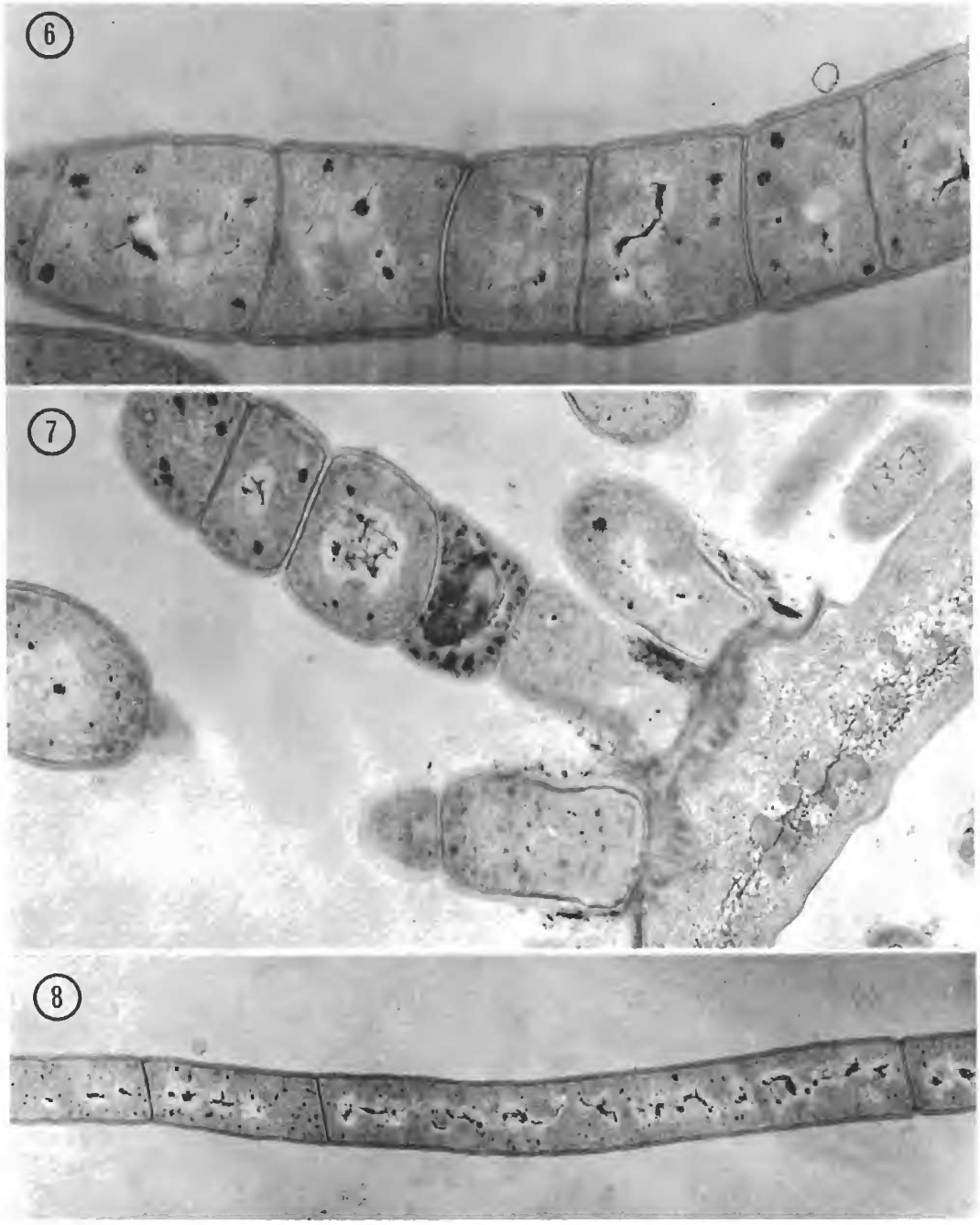
In a single attempt to culture our material, no more than eight cells developed in a chain. Numerous spherical cells, presumably gonidia, glided about on our "slide cultures." Filaments could not be demonstrated from liquid media when kept stationary or shaken or from a streaked agar plate.

Treatment

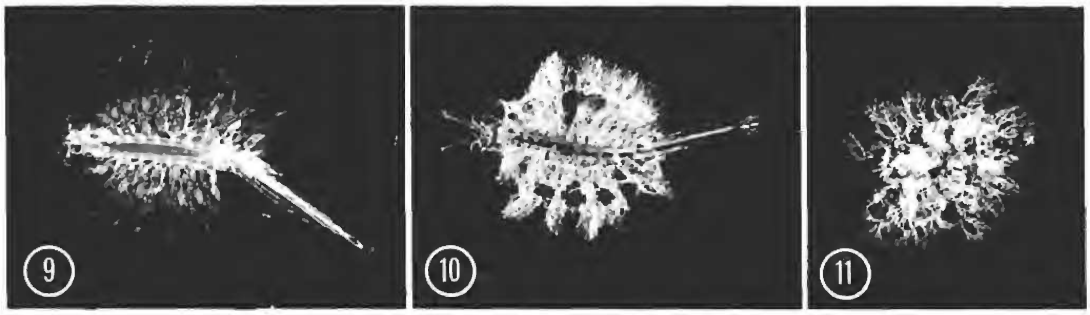
The average percentage per replicate of adult brine shrimp having sloughed bacterial mats and remaining free of *L. mucor* after exposure to a variety of treatments is listed in Table 1. Moderate variations in results for each concentration occurred among replicates. All treatments indicated some success for cure, but for reasons presented in the discussion



Figures 1–5. *Leucothrix mucor* on brine shrimp. (1) Moderate infestation showing abundant debris. (2) Close-up of setae showing attached bacterial colonies. (3) Close-up of debris and microorganisms. (4) High-power view of filaments showing granules. (5) Rosettes on brine shrimp seta.



Figures 6–8. Electron micrographs of filamentous organisms on brine shrimp. (6) Typical cells comprising a filament of *Leucothrix mucor*, x 32,800. (7) Holdfast attachment site for a rosette of *Leucothrix mucor*, x 29,300. Note assumed reproductive cell situated as second cell in middle filament. (8) Filament with some elongated cells, probably not *Leucothrix mucor*, x 16,100.



Figures 9–11. Photomicrographs of control shrimp and infestation of *Leucothrix mucor*. (9) Noninfested brine shrimp. (10) Brine shrimp moderately infested. (11) Sloughed mat and associated debris.

and in Table 1, treatment with 100 ppm terramycin seemed most practical.

DISCUSSION

Leucothrix mucor comprises a variety of over 30 strains (Raj 1977). Characterizations of these strains based on the combination of morphological features, biochemical assessments, and culturing ability are known for only a few. Because identification of our material is not positive and variations among strains are considerable, we tentatively consider our strain one of *L. mucor*.

Some strains of *L. mucor* differ in many respects from the one we report. Ours from the shrimp fits no clearly defined assemblage of characteristics. It has no sheath-like layer around the cell wall such as that reported by Anderson and Heffernan (1965), but neither do most strains. Brock and Conti (1969) showed irregularities and bleb-like extensions from the cell layers and a well-formed, thin, single-membrane, or middle layer (peptidoglycan), between the inner and outer double membranes, whereas the present form has relatively smooth membranes and no distinct middle layer. Abundant rosettes, such as those present on the brine shrimp, are not thought to be typical in rich nutritional regimes (Raj 1977). Steenbergen (San Diego State University, personal communication) has shown that a strain from the shrimp *Penaeus californiensis* has a uniquely different guanine-plus-cytosine ratio of deoxyribonucleic acid along with a lack of antigenic similarity when compared to other isolates. Consequently, that strain may not be *L. mucor*. The electrophoretic mobilities of enzymes were not studied in our material, but Kelly and Brock (1969) have shown significant differences in mobilities of two dehydrogenases from different strains. Strains also differ in their tolerance to salinity.

The status of the one or more species of *Thiothrix*, which closely resemble *L. mucor*, also remains uncertain because these anaerobic forms containing sulfur granules have not been consistently cultured. The composition of granules in our aerobic material was not analyzed, but their presence was inconsistent. Some filaments had them and others did not; moreover, a filament occasionally consisted of cells both with and without granules. Filaments from moribund shrimp, following three days of a gradual reduction in salinity, also possessed cells with and without granules. In any event, the presence and absence of granules did not provide evidence indicating the existence of two separate species or strains.

The holdfast did not spread out over the host's cuticle nor did it penetrate extensively. Couch (1978) suggested that the assumed mucoid substance of this holdfast might coat the gills of penaeid shrimp and block gas diffusion. We found that the cuticle became deformed at the attachment site. The holdfast of a strain of *L. mucor* on the peritrich *Zoothamnium* sp. infesting the gills of penaeid shrimp has been observed by Foster et al. (1978) to penetrate the stalk of the ciliate and to spread out into the extracellular fibrillar matrix. *Leucothrix mucor*, on occasion, probably penetrates several organisms or their products. It entangled the ciliate *Epistylis* sp. on fishes in low-salinity habitats (Overstreet and Howse 1977), but the filamentous organism (0.3 μm wide) within the stalk was definitely not *L. mucor* as implied by those authors (in their Figure 31).

In the natural environment, many crustaceans remain free of infestations by preening themselves. Bauer (1977) showed this by ablating the third maxillipeds of the shrimp *Heptacarpus pictus* so that it could not groom its antennules. The antennules of these test individuals became heavily fouled with *Leucothrix* sp.

Leucothrix mucor infests *Artemia salina* and many other crustaceans extensively when the medium is rich with nutrients. Consequently, rearing facilities foster infestations. According to J. A. Quick, Jr. (Dow Chemical Company, personal communication), *L. mucor* infests the shrimp only when the medium is enriched, even though shrimp populations exceed 100 per liter. Consequently, when practicality prevents culturing crustaceans by decreasing the nutrient levels, treating the system with chemicals should be considered. When contaminating rearing facilities, several strains of *L. mucor* can kill animals' hosts. Lightner et al. (1975) postulated that heavy infestations on penaeid shrimps caused

hypoxic conditions for the shrimp which thereby weakened or killed them, especially those shrimp molting or already in low-oxygen conditions.

In all the treatments tested (Table 1), the entire infestation on an individual sloughed as one mat. A mat involving some appendages and setae can be seen in Figure 11. Sloughed mats seemed to remain attached to a complete or partial molt involving the infested regions. Follow-up attempts to acquire mats for ultrastructural analysis of the holdfast and cuticle were unsuccessful. Such an investigation, however, seems desirable because sloughing often occurred within 1 to 2 hours after treatment.

The 2-day treatment with 100 ppm terramycin seemed the most desirable. In fact, introduction of 100 ppm into the culture tank eliminated the organism permanently, suggesting that the drug killed the parasite. The only other reported use of terramycin in controlling *L. mucor* was by Sandifer and Smith (1976) who obtained inconsistent results using 1-hour dips of concentrations up to 30 ppm. Their strain infested reared juveniles of *Macrobrachium rosenbergi* (the Malaysian prawn) in salinities of 12 to 13 ppt. We found an increasing percentage of cured individuals and a decrease in mortality as we increased the dose to 100 ppm. A concentration of 200 ppm produced results similar to those of 100 ppm.

Treatments other than terramycin had a variety of drawbacks. Rapidly decreasing the salinity from 50 to 10 ppt was successful, but the shrimp cannot survive and reproduce in low-salinity conditions for extended periods. Consequently, bacteria in the system would presumably reinfect the shrimp if the salinity were increased or if the shrimp from water with high-salt content were removed, treated, and returned. Moreover, when gradually decreasing the salinity to 10 ppt over 48 hours, only 2 of 17 shrimp sloughed their bacterial mats.

When under stress from many chemicals for time periods of various lengths, the brine shrimp is hardy. For example, specimens could withstand more than 10 minutes of 3% glutaraldehyde or 10% formalin and then live for at least 1 hour if transferred to normal sea water. On the other hand, even low concentrations of certain chemicals caused pathological responses in shrimp. Both potassium permanganate and formalin, used as indicated for treatments, caused shrimp to twirl, a continuous orbital movement from the surface to the bottom of the water column. Most host deaths caused by those compounds occurred during the first day, whereas those recorded in other treatments except Cutrine-plus® occurred gradually. Sandifer and Smith (1976) noted heavy mortality of prawns with $KMNO_4$ for long and short exposures, and Lightner (1977) reported a potential for severe gill damage in penaeid shrimp following a 1-hour treatment of 10 ppm. In our material, we noted blackened gills.

Twirling occurred in all treatments except terramycin, with that behavior most pronounced in high concentrations of cutrine and least pronounced in nitrofurazone. Nitrofurazone appeared to be the second-best treatment, but was tested for 6 hours only and should be investigated further.

The algacide Cutrine-plus (a chelated copper compound), while an effective drug for brine shrimp in a bath for up to 4 hours, caused many mortalities during 48-hour exposures, even at 0.5 ppm of the commercial product. In addition to twirling, those shrimp exposed to Cutrine-plus for less than 4 hours at 100 ppm also rotated in a spiral around their own axes. Lightner and Supplee (1976) also noted a toxic response by the California brown shrimp to that drug. In order for those authors to increase biomass and decrease mortality of that shrimp, they introduced 0.1 ppm Cutrine weekly for a 24-hour period in a flow-through system. A few other treatments have been tested with a variety of success (Lightner 1977, Sindermann 1977).

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